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Lyophilization of Human Platelet and Study of its Aggregability

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Abstract

Human platelet concentrates are usually stored in blood banks for 5 days, after expiration date they are discarded. Human lyophilized platelet is one of the approaches to preserve the platelets for long time storage. In our study trehalose was used as a stabilizer to protect the platelets during freezing and drying conditions.Platelet rich plasma was prepared as a source of platelet concentrate.Washed platelets were incubated in the presence of trehalose (40 mM /l) trehalose for 4 h at 37°C. After lyophilization. prehydration and rehydration stages, the response of lyophilized human platelets to the agonists such as ADP, ristositin and arachidonic acid were measured by an aggregometer instrument. Aggregation of platelets with ADP, ristositin and arachidonic acid showed 75.5%, 55.9% and 17.3% activity respectively. Clot formation at 37°C was clearly observed within 5 min after addition of these agonists to rehydrated lyophilized platelets.Our study showed that this method of preserving platelets with internal trehalose loading is possible in the dried state for storage at room temperature but for its quality improvement further investigation is required.

Keywords: Lyophilization, trehalose, platelet aggregation.

Introduction

Platelets are component of the blood that are critical for maintenance of hemostasis. They play an essential role to clot formation, wound healing and proper maintenance of blood vessels. Blood transfusion centers are under considerable pressure to produce platelets concentration for transfusion. Due to the short life span of human platelets using standard blood banking procedure, currently at 22°C in most blood banks, human platelets are maintained in liquid solutions at room temperature for only 5 days and then thrown away if not transfused within that period. Many approaches have been investigated experimentally to produce new hemostatically active platelet products that are capable of long-term storage [1-6]. Lyophilized platelet product which is one of the best choice can play a critical role in treatment of tendonitis [7], wound healing [8], hepatocyte proliferation [9] and control bleeding in animals [10-13].

The use of protective carbohydrate for preserving platelets during lyophilization has been investigated before in response to environmental stressors including chilling, freezing and drying [14-17]. Carbohydrates such as trehalose which is found at high concentration in a wide variety of organisms have an especial ability to stabilize platelets during freeze-drying process [17-18]. The principle mechanism of preservation during freezing and drying stress is mediated by direct bonding of the hydroxyl groups of these carbohydrates to membrane surfaces with creating water replacement structure that holds native conformation in place [18].

In this paper we describe platelet lyophilization process by using trehalose as a stabilizer to prepare functional freeze-dried platelets with aggregation capability.

Materials and Methods

Materials

Sodium chloride, potassium chloride, magnesium chloride, imidazole and HEPES were supplied from Merck Co. (Germany). Human serum albumin solution 20% was obtained from Biotest (Germany). Prostaglandin E1 and EGTA were purchased from Sigma-Aldrich (USA). All chemicals were of analytical grade. Human serum albumin was used without further purification.

Methods

Platelet Rich Plasma Preparation

Whole blood was obtained from Vesal Blood Transfusion Center (Vesal, Tehran) and then Platelet rich plasma (PRP) was prepared by centrifugation (2050 g, 4 min) and aggregation tests were performed as soon as possible.

Lyophilization process

PRP was centrifuged for 5 min at 500 g to remove leukocytes. The supernatant containing platelet was washed (600 g, 10 min) with washing buffer (100 mmol/L NaCl, 10 mmol/L KCl, 10 mmol/L EGTA, 10 mmol/L Imidazol, 10 µg/mL PGE1, pH:6.8). Washed platelet in a concentration of 0.8-1x10 ^{9} plt/mL were incubated at 37°C for 4 h in loading buffer (washing buffer in the presence of 40 mmol/L trehalose). The samples were stirred every 1 h during incubation. Platelets were pelleted and resuspended in lyophilization buffer (9.5 mmol/L Hepes,142.5 mmol/L NaCl, 4.8 mmol/L KCl, 1mmol/L $MgCl_2$, 30 mmol/L Trehalose, 1% human serum albumin, pH:6.8). Platelet concentrates were transferred in freezer -80C° with freezing rate -1C°/min for 90 minutes. After freezing, the frozen platelet solution were transferred to lyophilizator (VaCo 5-II, Zirbus Technology) under100 mili Torr vaccum pressure for 16 h. Freeze dried platelet concentrates were prehydrated for approximately 2 hours in a closed box with moisture-saturated air at 37°C. Vials containing freeze dried platelet rehydrated in 4 ml platelet poor plasma/water (2/1v/v).

Aggregation tests

For aggregation tests platelet suspension were transferred to aggregation cuvettes with a magnetic stirrer and response of the platelet to the ADP, Ristositin and Arachidonic Acid agonists were measured by Helena aggrigometer under stirring condition within 5 min.

Results and Discussions

Response of Platelets to Platelet Agonists

The aggregation of PRP by adding ADP, arachidonic acid and ristocetin agonists was measured by aggregometry with the activity of 70.5%, 89.5% and 82.3% while aggregation activity in rehydrated lyophilized platelet was found 75.5, 55.9 and 17.3% respectively.

The results of aggregation tests for PRP and rehydrated lyophilized platelet at standard agonist concentrations were summarized in table I. According to these results, response of rehydrated lyophilized platelet to the agonists were lower than PRP that was used as a starting material. We did not find any reason, why ristocetin response was the lowest. In general it seems that, freezing, drying and rehydration steps can lower membrane aggregability of platelet during lyophilization process, but the platelets have retained their aggregation properties somedeal.

Table 1. Platelet aggregation test results (%) of platelet rich plasma and rehydrated lyophilized platelet with various agonists.

Type of agonist	ADP	Arachidonic acid	Ristocetin
Platelet rich plasma	89.5	70.5	88.2
Rehydrated lyophilized platelet	75.5	55.9	17.3

Clot Formation

Clot formation at 37°C was clearly observed within 5 min after addition of these agonists to rehydrated lyophilized platelets. Furthermore, when the supernatant was measured with the Coulter counter, we found that no platelets were left in suspension. This indicated that all platelets participated in platelet aggregates formation.

Trehalose Uptake by Platelets

The findings of this study supported our hypothesis that rehydrated freeze-dried platelet concentrate is functional and can respond to the various agonists during aggregation tests. In oue experiment trehalose-loaded platelets were successfully freeze-dried, with relatively favorite recovery of intact platelets. Of course there is a major challenge to introduce sugar into the cell at high concentration. Current efforts helped at solving this problem include: (1) biosynthesis of trehalose by expression of trehalose genes in cells [18], (2) with creation of pores in the membrane [19], (3) taking advantage of the high permeability of membranes at the phase transition temperature [20], (4) introducing trehalose into the cytosol of human platelets with endocytosis pathway at higher temperature (above 25°C) which was discovered as a simple method by Wolkers et al [15]. In our study this simple method was used and platelets were loaded in the presence of 40 mM external trehalose at 37°C for 4 h. With this method trehalose is rapidly taken up by human platelets with loading efficiencies of 50% or greater [15].

Prehydration and Rehydration Step

The beneficial effects of pre-hydration and rehydration steps have been well studied [21-24]. According to the recent study [24], 1.5 h of prehydration had better effect than 3.5 h and there was no difference between 35°C and 37°C in preyhdration temperature. This study also showed that platelet poor plasma behaved better than phosphate-buffer saline as a rehydration solution. In our study, samples were settled in 2 h at 37°C during prehydration step and reconstituted with diluted platelet poor plasma for rehydration solution.

Conclusions

The findings of this study supported our hypothesis that rehydrated freeze-dried platelet concentrate is functional and can respond to the various agonists during aggregation tests.

In general, our study showed that this method of preserving platelets in the dried state is possible for storage at room temperature with using Iranian PRP units. However, trehalose loading efficiency, lyophilization process and proper storage conditions of the freeze-dried platelet need to be further investigated for optimizing process and improve aggregability of lyophilized platelet.

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